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ORIGINAL ARTICLE

Bacterial Cell Envelope Lysis and Hemotoxicity of Peptides Previously isolated From African Catfish, Clarias gariepinus

Hedmon Okella*[a], Clement Olusoji Ajayi[a], Hilda Ikiriza[a], Andrew Glory Mtewa[e], Bruhan Kaggwa[a,c], Jacqueline Aber[a,d], Charles Drago Kato[b], Patrick Ogwang Engeu[a]

[a]Pharm-Biotechnology and Traditional Medicine Centre, Mbarara University of Science and Technology, Mbarara, Uganda, [b]College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, Kampala, Uganda, [c]Department of Pharmacy, College of Health Sciences, Makerere University, Kampala, Uganda, [d]Department of Pharmacy, Faculty of Medicine, Gulu University, Gulu, Uganda, [e]Chemistry Section, Malawi Institute of Technology, Malawi University of Science and Technology, Limbe, Malawi

Correspondence to Hedmon Okella (hokella@std.must.ac.ug)

ABSTRACT

Background: The skin mucus layer of fish is endowed with biologics including, Antimicrobial peptides (AMPs) that offer a first line of defence against pathogens. Such peptides can either inhibit bacterial growth or completely kill the bacteria and hence are regarded as a viable alternative to traditional antibiotics, in addressing the ever-increasing incidences of antimicrobial resistance. However, one of the major hurdles to AMPs use is their poor haemolytic profile. As a result, a thorough evaluation of prospective AMPs' bacterial cell membrane disruption and hemolytic potentials in the early phases of drug discovery is critical. The current study presented cell membrane destruction as well as hemo-compatibility of antimicrobial peptides previously isolated from skin mucus of African catfish, *Clarias gariepinus*.

Methods: A previously isolated antimicrobial peptide in the skin mucus of African catfish, *C. gariepinus* were profiled using 15% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The electrical conductivity and alkaline phosphatase assays were utilised to measure bacterial cell envelope lysis activity as a classical mode of action of the antimicrobial peptides. Afterwards, fresh Rabbit blood cells were then utilised for in vitro hemolytic assay.

Results: The peptides were found to be about 5 kDa molecular weight with, ability to damage the bacterial cell envelope causing significant leakage in periplasmic alkaline phosphatase enzyme and cytoplasmic electrolytes. Even at the highest peptide extract concentration of 100 µg/mL, no significant hemolysis was observed on the fresh rabbit blood cells [3.63%;P>.05], signifying their safety on normal mammalian cells.

Conclusion: The findings of this study pointed out that antimicrobial peptides in skin mucus of *C. gariepinus* are potentially safe source of antimicrobial drug leads; however, further studies are still required to search for possibly maximum dose that is safe to host cells but still effective against infecting bacteria.

INTRODUCTION

Pathogenic bacteria, fungi, viruses and protozoa flourish in the same aquatic ecosystem in which fish live.[1] Such habitats predispose fish to higher infection risks compared to terrestrial vertebrates,[2,3]thereby calling for a more effective natural defence mechanism. Given their underdeveloped adaptive immunity, fish mostly utilise a more complex innate defence mechanism comprising three major components: physical, phagocytic cells and chemical mediators.[4,5] Over decades, it has been demonstrated that the fish mucus layer on the skin, gills, nose and gut remains the principal first-line physical defence against infections.[6,7] The mucus layer comprises a cocktail of biologics including peptides, acute phase proteins, glycoproteins, enzymes, immunoglobulin, lectins and hemolysin that are essential in contributing potential leads in the field of drug discovery.[8,9] However, the aquatic exploration has largely been for food sourcing with far less attention to fish-derived drug leads. This has left little known about the toxicological profiles and bactericidal efficacy of fish-derived antimicrobial peptides. Yet, exploring such may be of relevance to new alternative antimicrobial drug candidates in the era of new antimicrobial drug search.[10]

To this effect, the fish skin mucus has increasingly enthralled the search for new potential bactericidal drug candidates. Natively available fish biologics are gradually gaining pursuits as potential therapeutic candidates, due to their safety, low cost of production and rapid mode action[11].

Accordingly, several studies have investigated the antimicrobial activity of different fish species, notable examples include antimicrobial activity of skin mucus extracts of *Hypophthalmichthys nobilis*,[12,13] *Clarias batrachus*,[14–16] *Heteropneustes fossilis* and *Clarias batrachus*,[17] *Channa striatus*,[18] *Catla catla*,[19] *Rutilus frisii*,[20] *Periophthalmodon schlosseri*[21] and *Anabas testudineus*[22] among others.

However, studies on the antimicrobial potentials of *Clarias gariepinus* inhabiting any of the three major lakes of Uganda have not been reported. Besides, the bacterial cell lytic activity just like hemo-compatibility of such peptide extracted from the skin mucus of *C. gariepinus* in the African region remains unknown. The current work builds on a previous study where antimicrobial peptides from the skin mucus of the African catfish, *C. gariepinus* sourced from Lake Albert, Uganda were isolated.[23] In fact, the previous study was only limited to Sephadex G-25 peptide isolation. Therefore, in the quest for potential drug leads in the era of antimicrobial resistance, the current study presents the first report on bacterial cell envelope disruption and hemolytic profiles of such peptides.

MATERIALS AND METHODS

Preparation of antimicrobial peptides

Two lyophilized archived antimicrobial peptide fractions (Peak I and Peak II) previously isolated from 24 live mature *C. gariepinus* (Figure 1) in the family *Clariidae* (mean weight 300.50±5.98g, mean length 30.60±2.11 cm) using Sephadex G-25 gel filtration chromatography,[23] were utilised in the present study. Guided by our previous study, only fractions (Peak I) with demonstrable antimicrobial activity *E. coli* [(MIC: 0.31±0.16 µg/ml) and *S. aureus* (MIC: 1.99±0.13 µg/ml)][23] and clear band upon resolving on a 15 (%) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was considered. In addition, only the peptides extracted through Solid Phase Extraction (SPE) technique prior to Sephadex G25 bio-guided fractionation were employed in this study. This was due to the fact that cartridge's solid-phase hydrophobic matrix optimally captures the hydrophobic peptides that are later recovered through organic solvent systems.[24]

Peptide Profiling

To establish the peptide profiles, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was run as described by Laemmli with minor modifications.[25] A 15% separating gel and 4% stacking gel was used. Here, 0.01 g of lyophilized Peak I fractions and 0.01 g of lyophilized C_{18} SPE elute were dissolved in 50 µl deionized water, respectively. Thereafter, 40 µl of samples and 40 µl of sample loading buffer [12% SDS (w/v), 6% mercaptoethanol (v/v), 30% glycerol (w/v), 0.05% Coomassie blue G-250, 150 mm Tris/HCl, pH 7.0] were mixed, boiled for 5 minutes at 100 °C utilising heating block (Biobase, Shangdon, China). Then, 10 µl of the samples were then loaded into each well of the gel. Later, 8 µl of pre-stained SDS-PAGE standard markers (Thermo Fisher Scientific, Waltham, USA) were included to estimate the molecular mass of the proteins.

Electrophoresis was run at a constant voltage of 150 V for 55 minutes, until the dye front migrated to 2 cm from the bottom of the gel (Bio-Rad, Hercules, USA). Later, the gel was directly stained in 100 ml staining solution (1 g Coomassie R250, 30 ml, methanol, 65 ml deionized water, 50 ml acetic acid) for 4 hours.[26] Peptides were then directly visualized in distaining solution I (10 ml methanol, 10 ml Glacial acetic acid, and 80 ml deionized water) at 55 rpm rotation at room temperature; 25 °C (Edmund Buhler GmbH, Bodelshausen, Germany), until fairly clear bands were observed.

Alkaline Phosphatase Activity

Cell wall destruction was determined by measuring Alkaline Phosphatase Activity (ALP). An alkaline phosphatase assay kit (QuantiChrom BioAssay Systems, Hayward, USA) with *p*-nitrophenylphosphate (*p*NPP) as a substrate was used to measure the ALP activity of the cell lysate.[27] Briefly, 12 hour cultured *E. coli* were dissolved in fresh sterile medium to 106 CFU/ml. About 5 mg/ml of laboratory prepared antimicrobial peptide in 0.01 M Phosphate Buffer Solution (PBS) was added to the medium and cultured at 37°C (Heraeus, Hanau, Germany) for 30 minutes. The reaction mixture (1 ml) was then collected every hour, centrifuged at 5000 x *g* (Eppendorf, Dubai, UAE) for the next 20 hours. Thereafter, 150 µl of disodium *p*-nitrophenyl phosphate substrate buffer was added in the 50 µl supernatant and the mixture incubated in a 40 °C water bath (Grants, Cambridge-shire, UK) for 4 minutes. Absorbance was then measured at 405 nm using Microtiter plate reader (Biochrom, Cambridge, England) while 0.01 M PBS was used as a negative control. The experiment was carried out in triplicate before the Mean and Standard Error of Mean (SEM) were then calculated.

Electrical Conductivity Detection

Electrical conductivity of cytoplasmic fluid was determined as previously described by Lee et al.[28] Here, a 12 hour cultured *E. coli* was dissolved in fresh sterile medium to 106 CFU/ml. Thereafter, 0.05 mg/ml of antimicrobial peptides in 0.01 M PBS was added and cultured at 37°C (Heraeus, Hanau, Germany) for 20 minutes. The culture mixture (1 ml) was then collected every hour, centrifuged at 5000 x *g* (Eppendorf, Dubai, UAE) for the next 20 hours. The electrical conductivity was detected with Seven Go Duo SG-23 digital conductivity meter (Mettler-Toledo, Columbus, USA). The experiment was carried out in triplicate before the Mean and Standard Error of Mean (SEM) was then calculated, taking 0.01 M PBS as a negative control.

FIGURE 1. African Catfish

Clarias gariepinus (Burchell, 1822). Common name, African Catfish; Lango, Rec Lango/Twang; Luganda, Semutundu; Alur, Nyaii; Kiswahili, Kambale. A benthopelagic freshwater scaleless fish, with four pairs of slender, whisker-like sensory organs (barbel) near the mouth. An original photo by Hedmon Okella

Hemolytic Activity Testing

Hemolytic activity was assayed with a modified Rabbit blood cells method described by Lin and others.[29] Briefly,

fresh Rabbit blood cells were obtained by centrifuging whole blood from live rabbit in EDTA-coated Vacutainer (Becton & Dickinson, New Jersey, USA) in a refrigerated microfuge (Eppendorf, Dubai, UAE) at 211 x *g* for 5 min at room temperature (25 °C). Blood cells were washed three times with PBS, and then diluted with PBS to a blood cell concentration of approximately 10% (v/v).

A portion of the Rabbit blood cell suspension (500 µl) was transferred to six micro-centrifuge tubes (CellTreat, Massachusetts, USA), and mixed with 500 µL of antimicrobial peptide extract solution in 0.01 M PBS at the desired concentrations (1, 20, 40, 60, 80 and 100 µg/ml). The tubes were then incubated at 37°C (Heraeus, Hanau, Germany) to induce hemolysis. After 30 minutes of incubation, non-hemolysed blood cells were separated by centrifugation at 211 x *g* for 5 minutes at room temperature. Aliquots (100 µl) of the supernatant were transferred to a 96-well plate (Corning, New York, USA), and hemoglobin release was monitored by measuring the absorbance of the supernatant at 540 nm using microtiter plate reader (Biochrom, Cambridge, England). A blood cell solution treated with 1% Triton X-100 (to induce 100% lysis) was employed as a positive control, and an untreated blood cell suspension in 0.01 M PBS alone was used as negative control. Each assay was performed in triplicate for three independent experiments, and data were expressed as the mean and SEM. The percentage of hemolysis was calculated using the following formula[30]

Hemolysis (%)= ((Ae-An)/(Ap-An)) x 100

Where *Ae* is Absorbance of the extracts, *Ap* is the Absorbance of the positive control and *An* is the absorbance of the Negative control.

Statistical Analysis

Tableau Software v2019.4(Tableau, Seattle, U.S.A) was used to present data. All experiments were carried out in triplicate and expressed as Mean and Standard Error of Mean (SEM) using Prism 5.0 Statistical software (GraphPad, San Diego, U.S.A) and SPSS v16.0 (IBM, Chicago, U.S.A) were used to compare the means, in which a one-sample T-test was performed to determine the significance of the hemolytic activity of fish-derived antimicrobial peptides on fresh mammalian blood cells.

Ethical Consideration

Ethical approval and permission to conduct this study was obtained from the Mbarara University of Science and Technology Research Ethics Committee (22/11-18) and registered with the Uganda National Council for Science and Technology (HS449ES).

RESULTS

Study selection and Characteristics

In the present study, lyophilized Sephadex G-25 isolated antimicrobial peptides belonging to distinct peaks two peaks (Peak I and Peak II),[23] were utilised. Sephadex G-25 chromatographic resin was used and two prominent peaks (peaks I and II) were observed with absorbance measured at 280 nm. Only Peak I antimicrobial peptides demonstrated an antimicrobial activity with a MIC of 0.31 ± 0.16 and 1.99 ± 0.13 µg/ml on *E. coli* and *S. aureus*, respectively, whereas no antimicrobial activity was detected in peak II. The present study therefore, utilised only peak I archived samples of our previous study.[23]

Peptide Profiling

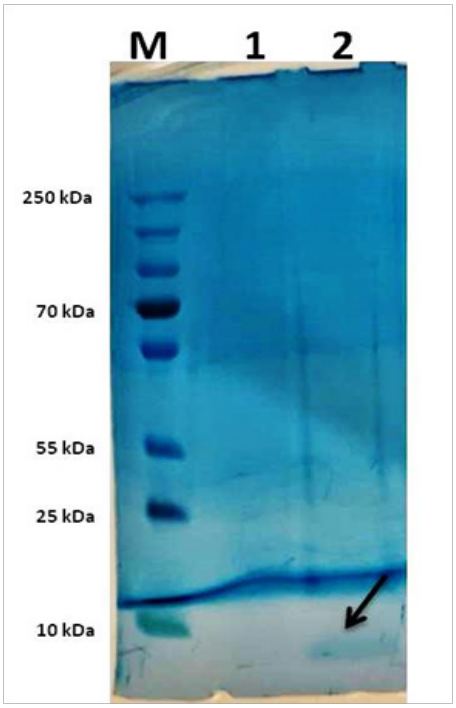
The SDS-PAGE peptide profiles of the previously extracted peptides from the skin mucus of African catfish are shown in Figure 2. Low molecular weight peptides (about 5 kDa) were observed as a clear band for the Sephadex G-25 purified extract unlike in the case of the 5 kDa-ultrafiltered C18 SPE elute.

Alkaline Phosphatase Activity and Electrical Conductivity

Throughout the culture process, the alkaline phosphatase content in the control group (0.01 M PBS), remained at a low level (below 5 iu/l), indicating the cell wall was intact (Figure 3a). In the antimicrobial peptide treated group, the alkaline phosphatase activity increased exponentially after an hour of treatment, as more alkaline phosphatase enzyme continues to leak out of the periplasm. Figure 3b showed the results of conductivity measurements.

Conductivity of the control group (0.01 M PBS) remained relatively stable at low level throughout the culture process. On the other hand, just after an hour of treatment with the antimicrobial peptides, the electrical conductivity was elevated significantly when compared to the control group, as the large volumes of the electrolyte continues to leak out of the cytoplasm during the culturing process.

FIGURE 2. A 15 (%) SDS-PAGE of peptides extracted from African catfish



The SDS-PAGE molecular marker lane (M), 5 kDa-ultrafiltered C18 SPE elute (1), C18 SPE and Sephadex G-25 isolated peptides (2). The extracted peptides were about 5 kDa.

Hemolytic activity of Antimicrobial Peptides

When the peptide extracts of *C. gariepinus* skin mucus were incubated with fresh rabbit blood cells, the percentage hemolysis increased with increase in peptide concentration (Figure 4). However, the increase in hemolytic activity was not significant even at the maximum concentration (100 µg/ml; $P>.05$) at a zero percent hemolysis test value. This signifies the safety of the fish-derived antimicrobial peptides (tested concentrations) on the Rabbit blood cells.

DISCUSSION

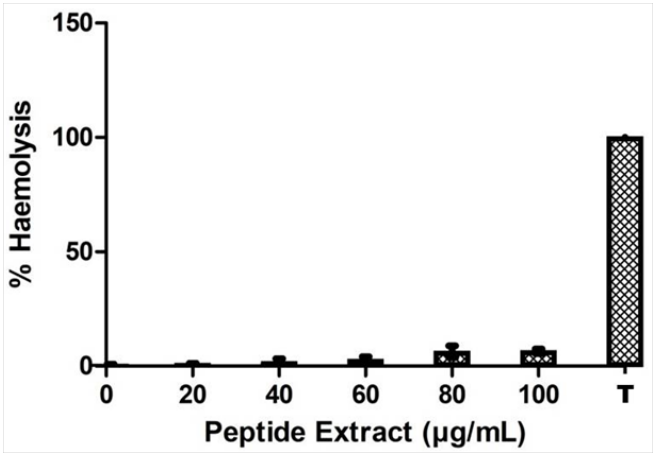
The present study demonstrated the capability of antimicrobial peptides extracted from *C. gariepinus* skin mucus in disrupting the bacterial cell wall and cell membrane. The previous study employed Solid Phase Extraction (SPE) of the antimicrobial peptides optimized the isolation of hydrophobic peptides. This is because solid phase of the SPE cartridge is a hydrophobic matrix that captures hydrophobic peptides that are later recovered by organic solvents after washing. Hydrophobicity is essential in bacterial cell membrane non-polar interactions.[31] Increased hydrophobicity up to the optimal threshold leads to enhanced ability of the peptides to associate with cellular hydrophobic lipid bilayer,[32] and most importantly, allows such peptide easily cross the membrane.[33,34] Much as, the present study was able to explore the action of these peptides on bacterial cell envelope (Figure 4), archived samples were utilized. Such samples are subject to deterioration. To mitigate this limitation, only lyophilized samples stored at 4 °C with clear band profiles on the SDS-PAGE and detected antimicrobial activity were considered for this study.

To gain insight into the mode of action of peptides, the bacterial cell envelope lysis of the *E. coli* was investigated by detecting the content of the extracellular alkaline phosphatase and electrolytes. The choice was guided by the fact that cell envelope damage is the commonest killing mechanism of peptides with antimicrobial or host defence potentials.[35] Alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphate esters in an alkaline environment, resulting in the formation of an organic radical and inorganic phosphate. In this study, a rapid increase in the alkaline phosphatase activity in the *E. coli* culture medium within an hour of peptide treatment was observed, this persisted till the 13th hour of treatment. This hourly trend monitoring is hinged on the fact that, antimicrobial peptides firstly targets the cell membrane,[36] and later interact electrostatically interact with negatively charged microbial cell membrane, prior to membrane destruction. Subsequently, the antimicrobial peptides may align parallel to the cell membrane like a carpet where it destroys the cell membrane in a 'detergent-like' manner,[37] or they penetrate the bilayer of the cell membrane as described in the pole model where their either form Toroidal pore/Wormhole[38] or aggregates into channel forming multimers.[39] Such sequence of events requires interval monitoring and an hour monitoring has been reported as ideal.[40]

Given that, alkaline phosphatase is localised between the cell membranes and cell walls of the bacteria[41] and and any disruption of the cell wall penetrability, leads to its discharge out of the bacteria. Therefore, *C. gariepinus* skin mucus peptides were capable of destroying the cell wall.

Similar increase in ALP activity, have previously reported for *Porphyra yezoensis* peptides[40] and Ornidazole (ORZ),[42] Linalool against *Pseudomonas fluorescens*,[43] Dihydromyricetin against Food-Borne Bacteria[44] and in AIEgen-Peptide Conjugate[45] among others, signifying bacterial cell wall destruction[46] and their potential application as far as sourcing of new antimicrobial drug candidates in the era of antimicrobial drug resistance is concerned.

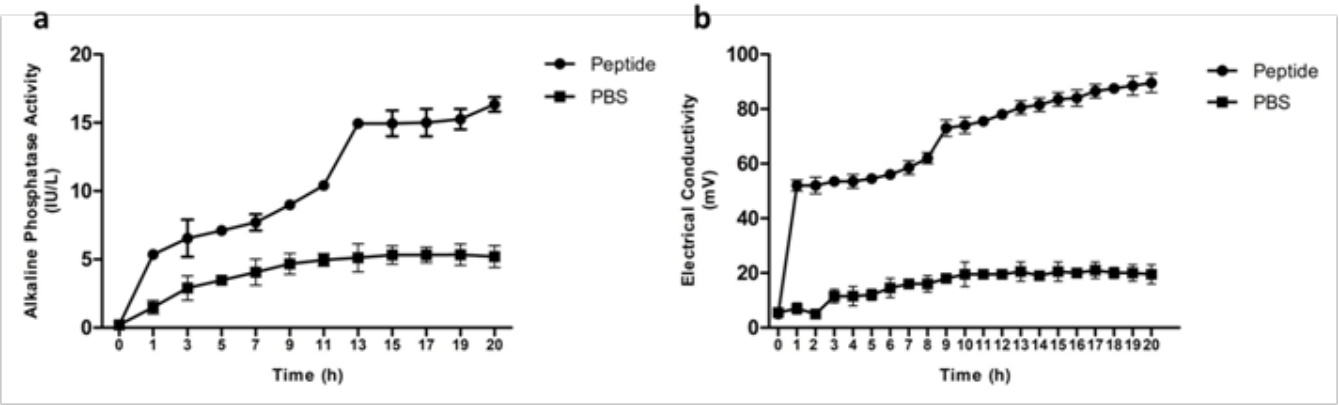
FIGURE 4. Percentage hemolysis of Rabbit blood cells by peptide extracts of C. gariepinus.



T-1% Triton X-100. The concentration of 1-100 µg/ml did not hemolyze the Rabbit blood cells. The data are expressed as Mean± SEM

Besides, cell wall distraction increases the cell membrane permeability resulting to cytoplasmic outflow and hence, a rise in extracellular electrolytes.[47,48] Monitoring of the electrical conductivity of the extracellular medium can therefore be employed as a measure of the bacterial cell membrane disruption.[49] In this study, within an hour of peptide treatment, the electrical conductivity of *E. coli* culture medium significantly increased suggesting the cell wall and cell membrane breakage amounting to cytoplasmic fluid outflow, cell collapse and death. The gradual increase in conductivity after an increase, is possibly due to the fact that majority of the cytoplasmic electrolytes are lost within the first hour of membrane shock. This in line with studies on OVTp12 peptide on *E.coli* and *S. aureus;*[50] Metallic oxide powders on *Candida albicans* NBRC1060, *Saccharomyces cerevisiae* NBRC1950, *Aspergillus niger* NBRC4067 and *Rhizopus stolonifer* NBRC4781;[51] *Porphyra yezoensis* peptides on *S. aureus;*[40] Black Paper Essential Oils (BPEO) on meat-borne *E.coli*[52] and antibiotics like Penicillin G, Nalidixic acid, Rifampicin on *Escherichia coli* 745 and *Staphylococcus aureus* 9779.[53] Moreover, cell damage as well ignites transient pore formation and freight of peptides into the cell for intracellular target interactions.[54] Much as, it would be essential to explore the interactions of the isolated peptides with the intracellular targets, it was beyond the scope of this study to explore peptide-intercellular target

FIGURE 3: Effect of Antimicrobial Peptide Treatment on Cell Wall and Cell Membrane Integrity



(a) Alkaline phosphatase activity in the culture medium, (b) Electrical conductivity in culture medium. PBS-0.01 M Phosphate Buffer Solution. Both the Alkaline phosphatase activity and Electrical conductivity were measured each hour for 20 hours. The data are expressed as Mean±SEM.

interactions as dictated by the facility constrains.

Toxicity against mammalian cells is the chief drawback that impedes most peptides from penetrating the pharmaceutical market.[55,56] To this effect, preliminary assessment of any potential lead biologic or their sources for hemo-compatibility is worthy. In this study, *C. gariepinus* peptide extracts was found to be non-hemolytic even at the highest concentration assayed (100 µg/ml), signifying their potential safety towards mammalian red blood cells and hence, relevance in sourcing of novel antimicrobial drug leads. The maximum concentration assayed (100 µg/ml), was empirically arrived at based on the previous studies.[57,58] Non-hemotoxic findings have as well been demonstrated in the skin mucus extract of Striped Dwarf Catfish, *Mystus vittatus;*[57] *and* Marine Catfish, *Tachysurus Dussumieri.*[59]

To the contrary, the mucus extracts of Spotted sea catfish, *Arius maculatus;*[58]and venomous fish such as Stonefish, *Synanceia verrucosa;*[60] Lionfish, *Pterois volitans;*[61,62] Scorpionfish, *Scorpaena plumieri;*[63] Pufferfish, *Akifugu rubripes*[64] have been reported as hemotoxic and lethal. The biologics in the hemotoxic extracts penetrates the deeper hydrophobic core of the mammalian cell membrane, once their hydrophobicity exceeds the optimal threshold.[65] Therefore, the antimicrobial peptides of *C. gariepinus* were below the hydrophobicity threshold for the studied concentrations. However, due to facility limitations, we were unable to determine the optimal threshold hydrophobicity for the extracted peptides.

CONCLUSION

The present study for the first time unveiled the toxicity profiles and possible mode of action of the previously isolated African catfish antimicrobial peptides. The peptides demonstrated outstanding lytic activity on the bacterial cell envelope. Furthermore, they were non-hemolytic to normal mammalian blood cells. The current study therefore, fronts the novel source of safe and efficacious antimicrobial drug leads of potential applications to food, medicinal and pharmaceutical industries. However, we recommend the search for potentially maximum dose that is safe to the host cells but still effective against bacteria.

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